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# Which extractant should be used for the screening and isolation of antimicrobial components from plants?

J.N. Eloff

*Department of Pharmacology, University of Pretoria, Pretoria, South Africa*

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**Abstract**

Freeze dried and finely ground leaves of two plants with known antimicrobial activity, *Anthocleista grandiflora* and *Combretum erythrophyllum* were extracted with acetone, ethanol, methanol, methylenedichloride, methanol/chloroform/water and water at a 1 to 10 ratio in each case. The quantity and diversity of compounds extracted, number of inhibitors extracted, rate of extraction, toxicity in a bioassay, ease of removal of solvent and biological hazard were evaluated for each extractant. An arbitrary scoring system was developed to evaluate the above parameters for the different extractants. Acetone gave the best results with these plants with an arbitrary value of 102 followed by methanol/chloroform/water (81), methylene dichloride (79), methanol (71), ethanol (58) and water (47). Four five minute sequential extractions of very finely ground *A. grandiflora* shaking at a high rate extracted 97% of the total antimicrobial activity. © 1998 Elsevier Science Ireland Ltd.

*Keywords:* *Anthocleista grandiflora*; *Combretum erythrophyllum*; Acetone; Ethanol; Methanol; Methylenedichloride; Methanol/chloroform/water

## 1. Introduction

The World Health Organisation estimates that 80% of the people living in developing countries almost exclusively use traditional medicine. Medicinal plants form the principle component of traditional medicine. This means that in the order of 3300 million people use medicinal plants on a regular basis. Medicinal plants used in traditional medicine should therefore be studied for safety and efficacy (Farnsworth, 1994).

Medicinal components from plants also play an important role in conventional western medicine. In 1984, at least 25% of the prescription drugs issued in the USA and Canada were derived from or modelled after plant natural products (Farnsworth, 1984). In 1985, Farnsworth et al. identified 119 secondary plant metabolites that are used globally as drugs. It has been estimated that 14–28% of higher plant species are used medicinally, that only 15% of all angiosperms have been investigated chemically and that 74% of pharma-


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cologically-active plant derived components were discovered after following up on ethnomedical use of the plant (Farnsworth and Soejarto, 1991). Southern Africa contains ~10% of the worlds plant diversity, but relatively little chemical work has been done on medicinal plants from this region. Of the 300 plants investigated by Noristan, 31% showed marked activity, 48% were moderately active and 21% were inactive (Fourie et al., 1992).

The numbers of resistant strains of microbial pathogens are growing since penicillin resistant and multiresistant pneumococci caused a major problem in South African hospitals in 1977. Berkowitz (1995) calls the emerging of drug resistant bacteria a medical catastrophe. Leggiadro (1995) stated that effective regimens may not be available to treat some enterococcal isolates and that it is critically important to develop new antimicrobial compounds for these and other organisms before we enter the post-antibiotic era. New compounds inhibiting micro-organisms, such as benzoin and emetine have been isolated from plants (Cox, 1994). The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains.

For these reasons, there has been a substantial increase in the number of papers where authors screened plants for antimicrobial properties. From these papers it is clear that authors use different extractants varying from 80% ethanol (Vlietinck et al., 1995), methanol (Taylor et al., 1995), petroleum ether, chloroform, ethanol, methanol and water (Salie et al., 1996). After reviewing the re-awakening of pharmacognosy Cordell (1993) concluded that 'There is clearly substantial room for improvement in the extraction methodologies, given that there are a variety of techniques that could be used to prepare extracts. Farnsworth in Balick (1994), states that the biggest problem in drug development with plants is answering a very simple question: what kind of extract should we test?

Various solvents have been used to extract plant metabolites. Many scientists employ soxhlet extraction of dried plant material using solvents with increasing polarity, e.g. ether, petroleum ether, chloroform, ethyl acetate and ethanol. This works well for compounds that can withstand the temperature of the boiling solvent, but can not be used for thermolabile compounds. The problem can be overcome by extracting under reduced pressure, but it is difficult.

The choice of solvent depends also on what is intended with the extract. If extraction is to screen plants for antimicrobial components, the effect of the extractant on subsequent separation procedures is not important, but the extractant should not inhibit the bioassay procedure. If the plant material is extracted to isolate chemical components without using bioassay, toxicity of the solvent is not important because the solvent can be removed before subsequent isolation procedures.

In order to find an extractant that would be optimally useful both in the screening and isolation of antimicrobial components from plants, I decided to compare a number of extractants. After a scrutiny of the literature to determine which extractants were used, I initially compared ethanol used by the National Cancer Institute in the USA (Suffness and Douros, 1979), a 1:1 mixture of methanol and methylene dichloride (Balick, 1991) and a one-phase mixture of methanol, chloroform and water (12:5:3) (Bieleski and Turner, 1966).

Due to problems encountered with the subsequent treatment of extracts (Section 4), the following extractants were compared: acetone, ethanol (EtOH), methanol (MeOH), methylene dichloride (MDC), a mixture of chloroform, methanol, water (12:5:3) (MCW) and water. Preliminary work with dimethylsulfoxide, frequently used in microbial studies, was abandoned due to the high boiling point 189°C and because it was also more toxic to *Staphylococcus aureus* than acetone in the bioassay used (results not shown).

Fresh or dried plant material can be used as a source for secondary plant components. Most scientists working on the chemistry of secondary plant components have tended to use dried material for the following reasons: (i) There are fewer problems associated with the large scale extraction of dried plant material than with fresh mate-

rial; (ii) the time delay between collecting plant material and processing it makes it difficult to work with fresh material because differences in water content may affect solubility or subsequent separation by liquid–liquid extraction; (iii) the secondary metabolic plant components should be relatively stable especially if it is to be used as an antimicrobial agent; (iv) many, if not most plants are used in the dried form [or as aqueous extract] by traditional healers.

The two plant species used in this study *Anthocleista grandiflora* (Burch.) Sond. (Loganiaceae) and *Combretum erythrophyllum* (Afzel. ex R. Br.) Gilg. (Combretaceae) were identified as plants containing antimicrobial activity (Dr T.G. Fourie, personal communication) after an extensive screening of South African plants by Noristan (Fourie et al., 1992).

The following parameters were investigated with the different extractants: the quantity extracted, the rate of extraction, the diversity of different compounds extracted, the diversity of inhibitory compounds extracted, the ease of subsequent handling of the extracts, the toxicity of the solvent in the bioassay process, the potential health hazard of the extractants. The different solvents were compared by grading on a five point weighted scale.

## 2. Materials and method

### 2.1. Plant material

The Noristan scientists discovered that *Combretum erythrophyllum* (Burch.) Sond. and *Anthocleista grandiflora* (Afzel. ex R. Br.) Gilg. had some antibiotic activity, but did not follow up the preliminary results. Leaf material was collected from a tree in the Pretoria National Botanic Gardens (*C. erythrophyllum*) and a tree on the campus of the University of Pretoria (*A. grandiflora*). Plants were identified by the plant labels on the trees and confirmed by Professor A.E. van Wyk, Pretoria University and Professor G.F. Smith, National Botanical Institute, respectively.

### 2.2. Preparation of leaf material and extraction

Leaves were dried in the shade at room temperature or freeze dried. Stems and thick veins were removed. Leaves were ground to a fine powder in a Jankel and Künkel Model A10 mill. Leaves were initially extracted on a shaking machine with a 10:1 solvent to dry weight ratio for 4, 6 and 24 h. Later 0.5 g finely ground plant material was extracted with 5 ml of the extractant in a centrifuge tube and the extract was decanted after centrifuging at 300 × *g* for 5 min. The process was repeated three times. The extractants used were technical grade (Merck). For quantitative determination, solvent from extracts placed in preweighed glass vials were removed by a stream of air in a cold room or at room temperature.

### 2.3. Analysis of extracts

Thin layer chromatography (5 μl of 100 mg extract/ml solution) was on Merck TLC F254 plates with acetone/methylenedichloride 2/3 as eluant. This system separated components over a wide range of $R_f$ values and consequently polarities. More polar components were separated using 6:1 acetone:methylenedichloride. Separated components were visualised under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). In some case, plates were sprayed with 5% anisaldehyde in a 5% sulphuric acid in ethanol solution and heated at 100°C.

### 2.4. Bioassay

The bioautographic procedure described by Begue and Kline (1972) was used. Chromatography plates were dried overnight and sprayed with a concentrated suspension of actively growing cells of *Staphylococcus aureus* or *Pseudomonas aeruginosa* (both from ATCC), before incubating at 38°C in a chamber at 100% relative humidity. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma). Inhibition of growth was indicated by clear zones on the chromatogram after incubating for 1 h. Toxicity of solvents to *S. aureus* and *P. aeruginosa* were determined by serial dilution with medium to the level where growth was not inhibited.

## 3. Results and discussion

### 3.1. Drying of plant material

There was no difference in the toxicity level to the test organisms or the number of compounds extracted or $R_f$ values for both plants when comparing freeze dried and leaves dried at room temperature. The toxicity decreased and the number of compounds separated by TLC changed when the leaves were dried overnight at 105°.

### 3.2. Quantity extracted with initial extractants

In initial experiments with the two plants, the plant material was not ground finely and extraction took place over 4, 6 and 24 h on a rotating shaker. MCW extracted the most material followed by ethanol and MeOH/MDC in both plants. In both cases, MCW extracted components at the highest rate. MCW extracted a total of 45% of the dry weight of *A. grandiflora* and 35% of the dry weight of *C. erythrophyllum* in 24 h. Problems were encountered in the subsequent handling of large numbers of extracts with MCW as solvent due to: (i) The separation into two phases when concentrated; (ii) the low volatility of the water; and (iii) problems to dissolve the dried extracts in solvents used for TLC. Changes may also occur over such a long period of extraction.

### 3.3. Extraction period

Malone (1983) stated that very finely ground plant material suspended in an inert dosing vehicle, i.e. sterile 0.25% agar solution would mobilise from the rat peritoneal cavity into the blood almost as fast as if they had been injected in a soluble form. It may be possible to shorten the extraction period by grinding the leaves finer and by shaking at a very high rate for a short period. The average diameter of the particles of the plants that we ground in the mill was $\sim 10$ $\mu$m. After three 5 min extractions, 49% of the *A. grandiflora* and 38% of the *C. erythrophyllum* dry mass was extracted. These values were even higher than values obtained after 24 h in a shaking machine with less finely ground material.

### 3.4. Quantity and rate of extraction

This technique was then applied to all the extractants. The results are shown in Fig. 1. MCW and methanol extracted the most material, i.e. 34 and 33% and 23 and 24% of the dry weight of the two plants in the first extract. The next highest values was with water followed by EtOH, MDC and acetone which extracted similar quantities. The rate of extraction was the highest for acetone in the case of *C. erythrophyllum*, but in the case of *A. grandiflora* MeOH and MCW had the highest rate of extraction. To determine what percentage of the soluble components were extracted after three extractions, the process was repeated six times on *A. grandiflora* with acetone. The fourth, fifth and six extracts extracted an additional 8, 8 and 7% of the total quantity extracted. There was no apparent difference in the composition of any of the six extracts. Bioassay of the extracts using *S. aureus* by serial dilution to the level where growth was not inhibited indicated that the six sequential extractions extracted 50.7, 23.2, 13.5, 9.6, 2.9 and 0.5% of the total antimicrobial activity. Four 5 min extractions with acetone would extract practically all [$\sim 97.6\%$] the antimicrobial activity from *A. grandiflora*.

### 3.5. The diversity of components extracted

The larger the variety of compounds that are extracted by the extractant, the better the chance that biologically active components will also be extracted if a specific class of chemical component is not targeted. To investigate the number of components in the extracts, several TLC systems were investigated. To facilitate the use of biochromatography, neutral and volatile solvents were used. Good results were obtained with methylenedichloride–acetone mixtures. The extracts were separated by TLC. Separated components were viewed in visible light, under UV at 360 nm, by fluorescence quenching under 254 nm and after spraying with 5% anisaldehyde in a 5% sulphuric acid in ethanol solution and heating at 100°C.

A problem experienced with the MCW mixture is that it separated into two phases after concen-

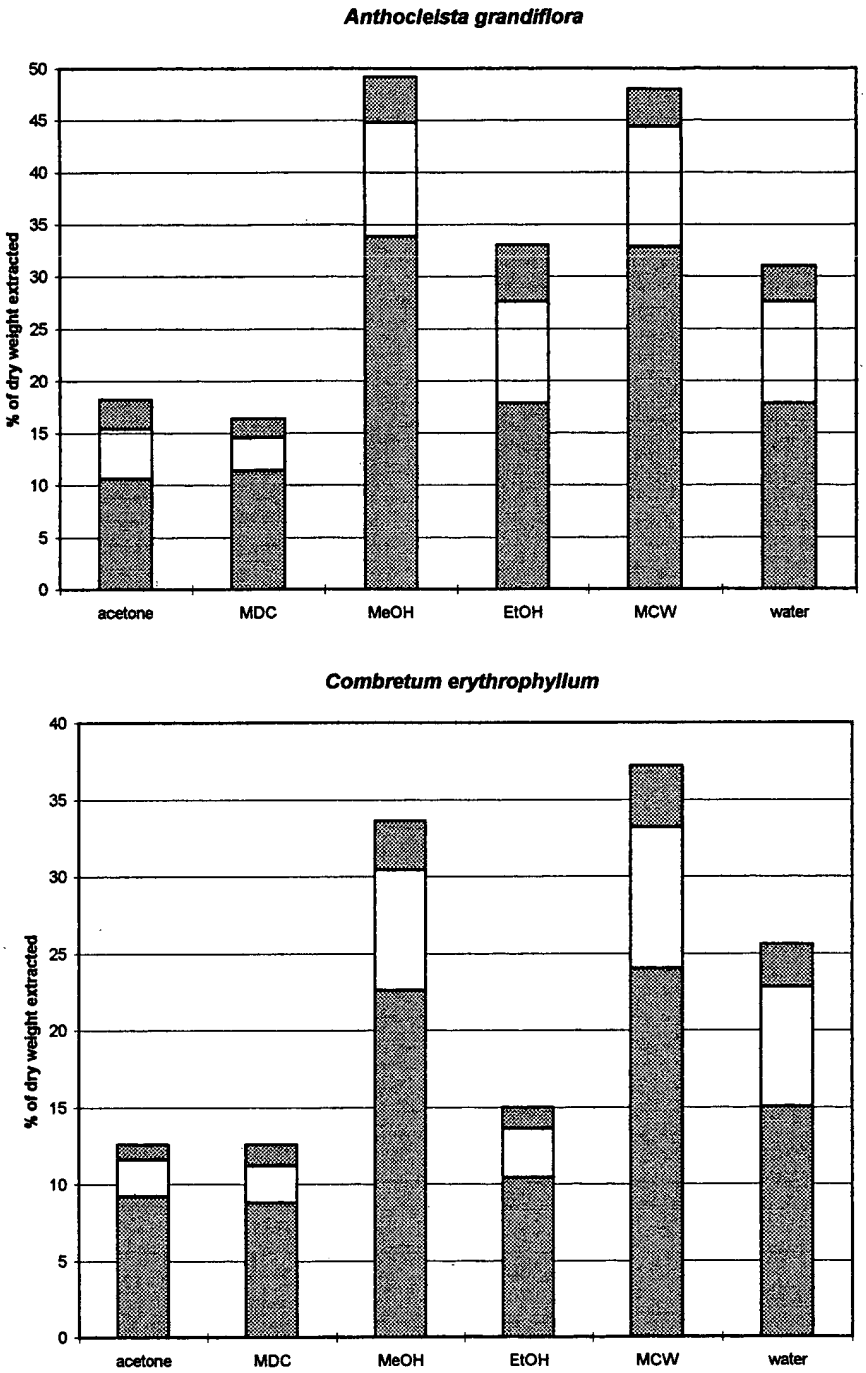


Fig. 1. Percentage of 0.5 g freeze dried material extracted after three extractions with 5 ml of acetone, methylene dichloride (MDC), methanol (MeOH), ethanol (EtOH), a mixture of chloroform, methanol, water (12:5:3) (MCW) and water. Value for first extraction at bottom, second in middle and third at top.

Table 1
The number of components visible on TLC chromatograms and number of inhibitors of *Staphylococcus aureus* growth from different extracts of *A. grandiflora* and *C. erythrophyllum* that separated in the bioautographic TLC system

| | *A. grandiflora* | | *C. erythrophyllum* | |
|---|---|---|---|---|
| | Components | Inhibitors | Components | Inhibitors |
| Acetone | 13 | 2 | 9 | 14 |
| MDC | 9 | 2 | 8 | 11 |
| MeOH | 11 | 2 | 9 | 9 |
| EtOH | 9 | 0 | 8 | 8 |
| MCW (chloroform) | 9 | 2 | 4 | 13 |
| MCW (water) | 8 | 0 | 9 | 1 |
| Water | 5 | 0 | 3 | 1 |

trating in a cold stream of air. Results are shown for the water and the chloroform fractions of the extract.

### 3.6. Number of inhibitors extracted

The results on the number of inhibitors present in the different extracts are summarised here and are discussed in more detail elsewhere. The best results were obtained with *S. aureus* as test organism (Table 1). A major part of the biological activity in the *C. erythrophyllum* extracts were also in the highly polar components that could only be moved to a degree from the origin of the chromatography plates by polar solvents. Acetone extracted both polar and non-polar inhibitors, as seen from biochromatograms using different solvent systems. The acetone extract had the lowest minimal inhibitory concentration for gram-positive bacteria (results not shown). Acetone extracted the largest number of different components and inhibitors from both plants (Table 1).

### 3.7. The ease of subsequent handling of the extract

The following difficulties were encountered with some of the extractants used: removing the solvents from a large number of fractions, freeze drying methanol extracts, concentrating MCW extracts, diluting MDC extracts with water before bio-assay, re-dissolving some of the extracts, decanting the supernatant after centrifuging the extract and toxicity during bioassay studies. The evaluation of the different extractants is listed in Table 2.

### 3.8. Summarising the results

No extractant was the best in all of the parameters tested. The different parameters are also not equally important to attaining the objectives. To compare the different extractants they were evaluated on a 5-point scale (5 best and 1 worst). The different parameters were also given different weighting based on a judgement of the importance of that parameter. The values on the 5-point scale were multiplied with the weighting (Table 3).

According to the parameters used and the weight given to the parameters, acetone was by far the best extractant followed by MCW and MDC that had similar values. The weight allocated to the different parameters is decidedly subjective, but is not likely that changes would effect the prime position of acetone because acetone scored the highest in all but one parameter. Next in line was MeOH, followed by EtOH and water. Apart from the results with EtOH, there were no major differences in the results for the different solvents between the two plants. There was a difference between EtOH and MeOH as extractant in most of the parameters measured, especially in the toxicity to the bioassay organism and biological hazard.

Table 2
Physical characteristics of the various solvents and inhibition of growth in bioassay

| | Boiling point | Volatility[a] | Flammability | Biological hazard | Toxicity to St. aureus[b] | Toxicity to Ps. aeruginosa[b] |
|---|---|---|---|---|---|---|
| Acetone | 56.5 | 35 | Flammable | | 28 | 28 |
| MDC | 39.8 | 38 | | Harmful | 17 | 13 |
| MeOH | 64.7 | 22 | Flammable | Very toxic | 7 | 4 |
| EtOH | 78.5 | 14 | Flammable | | 28 | 10 |
| MCW(chloroform) | 61.5 | 19 | | Harmful | 7 | 13 |
| Water | 100 | 1 | | | >38 | >38 |

[a] % of solvent evaporating within an hour.
[b] Highest % dilution at which growth was apparently not inhibited.

## 4. Conclusion

Because acetone dissolves many hydrophylic and lipophylic components from the two plants used, is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant. Fractions collected from a chromatography column dry off within a relatively short period in a cold room.

In this study, the use of the solvent for screening and for the isolation of active components was examined. If the aim of the research is to extract components for preparative work only without doing a bioassay of the extract, MCW is useful. Group separation by liquid–liquid extraction is already attainable by adding 1/6 of the volume chloroform and 1/4 of the volume water which cause a separation of lipophylic and hydrophylic components (Bieleski and Turner, 1966). In the case of *C. erythrophyllum*, the chloroform fraction contained many inhibiting components and few components visible after TLC.

If the intention is to screen a number of plants, acetone was much easier to use. The greatest advantages are the volatility, miscibility with polar and non-polar solvents and its relatively low toxicity to the test organisms. It is also clear that some difference occurs between plants. The antimicrobial components in these plants are more lipophylic and different results may be obtained with other plants. Due to the ease of handling extracts and fractions at a subsequent stage, acetone is probable preferable to MeOH, EtOH and water, even when more hydrophylic compounds

Table 3
Comparison of extractants on different parameters based on a five point scale (1–5) and with different weights allocated to the different parameters

| Parameter | Weight | Acetone | | EtOH | | MeOH | | MCW | | MDC | | Water | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | C | A | C | A | C | A | C | A | C | A | C |
| Quantity extracted | 3 | 6 | 3 | 9 | 6 | 12 | 12 | 12 | 12 | 3 | 3 | 9 | 9 |
| Rate of extraction | 3 | 12 | 15 | 12 | 12 | 12 | 12 | 12 | 12 | 15 | 15 | 9 | 9 |
| Number of compounds extracted | 5 | 20 | 20 | 10 | 15 | 15 | 20 | 10 | 15 | 10 | 15 | 5 | 5 |
| Number of inhibitors extracted | 5 | 20 | 20 | 0 | 10 | 20 | 15 | 20 | 20 | 20 | 15 | 0 | 0 |
| Toxicity in bioassay | 4 | 16 | 16 | 8 | 8 | 0 | 0 | 8 | 8 | 8 | 8 | 16 | 16 |
| Ease of removal | 5 | 20 | 20 | 5 | 5 | 10 | 10 | 10 | 10 | 20 | 20 | 0 | 0 |
| Hazardous to use | 2 | 8 | 8 | 8 | 8 | 2 | 2 | 6 | 6 | 6 | 6 | 8 | 8 |
| Total | | 102 | 102 | 52 | 64 | 71 | 71 | 78 | 83 | 79 | 79 | 47 | 47 |

A, results for *A. grandiflora*; C, results for *C. erythrophyllum*

are investigated because acetone also extracted highly polar components from these plants. The possible inefficiency in extracting hydrophylic components is more than offset by the ease of subsequent handling. It may be worthwhile comparing some of these extractants on other plants and on other plant parts to determine if a generalization can be made on the usefulness of acetone as an extractant.

Snyder and Kirkland (1979) grouped solvents into eight groups, depending on the dipole, as well as ability to act as H-donors or H-acceptors. Acetone forms part of group VIa. Other solvents in this same group that are miscible with water are dioxane and to a lesser degree ethylacetate and may be useful as extractants for dried plant material.

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